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CHEMICAL CARCINOGEN-INDUCED CHANGES IN TRNA METABOLISM  
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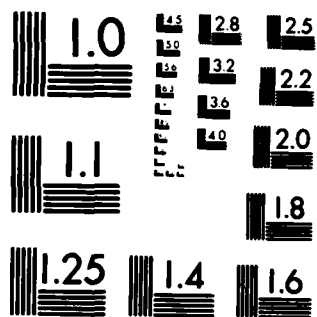
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CHEMICAL CARCINOGEN-INDUCED CHANGES IN  
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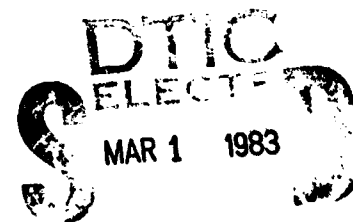
Ronald Trewyn  
Department of Physiological Chemistry

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) <b>✓ It was demonstrated that the nucleic acid catabolite 7-methylguanine (a major product of many carcinogen methylation reactions as well as a natural component of RNA) modulates tRNA modification by inducing queuine hypo- modification. Phorbol ester tumor promoters were found to induce similar tRNA changes immediately preceding major phenotypic alterations in cultured human cells. Culture conditions were defined which allow normal human cells to mimic <u>in vitro</u> transformation in the continuous presence of</b>		

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active phorbol esters; the characteristics of transformation induced include 5 to 10-fold increases in saturation density and anchorage-independent growth. Phorbol esters which are inactive as tumor promoters on mouse skin did not induce the phenotypic or biochemical changes in cultured human cells. Two-stage promotion was also demonstrated for certain agents (e.g. norharman and anthralin) subsequent to phorbol ester exposure. In addition, one or more new tRNA transglycosylases have been tentatively identified.

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## b. Research Objectives

The grant proposal submitted in 1980 tendered the hypothesis that changes in tRNA metabolism are required for the progression of cells through the stages of carcinogenesis. Following the induction of the carcinogenic process, gradual phenotypic modulation towards a less differentiated cellular state occurs. Alterations in tRNA modification and catabolism are known to commence soon after chemical carcinogen exposure, and the accruing changes in tRNA isoaccepting species may be involved in releasing the post-transcriptional controls over developmental gene expression.

At some early time after the induction of carcinogenesis it is proposed that there is an elevation of RNA methylation which likely leads to the increase in the levels of methylated RNA catabolites. These catabolites may modulate tRNA modification further, either by inhibiting modification (e.g. by blocking tRNA: queuine transglycosylase) or by acting as an alternative substrate. New tRNA isoaccepting species are generated by the changes in macromolecular modification and these species might translate different (e.g. fetal) mRNA's that are present. By this method, onco-developmental proteins would be synthesized. Some of the new proteins could be RNA methyltransferases and this would start the cycle over again at a more aberrant level. Finally, the stage of neoplastic transformation would be reached. We are examining the variations in RNA metabolism to determine if they are, in fact, involved in the dedifferentiation and progression of carcinogenesis.

## c. Status of the Research

Major progress has been made during the second year of this AFOSR sponsored project in establishing the role of altered tRNA metabolism in the expression of carcinogenesis. Of particular relevance to the molecular scheme hypothesized was our demonstration that the RNA catabolite 7-methylguanine can modulate tRNA modification by inducing queuine hypomodification (Elliott and Trewyn, 1982). 7-Methylguanine promotes changes in phenotypic expression in cultured mammalian cells, including neoplastic transformation. Under similar conditions, 7-methylguanine inhibits tRNA: queuine transglycosylase, the enzyme responsible for queuine insertion into the anticodon of tRNAs for aspartic acid, asparagine, histidine, and tyrosine. A role for the tRNA molecular changes in causing the cellular phenotypic changes has been proposed (Trewyn et al., 1982).

The only tRNA modification known to occur by a transglycosylase-type reaction is the one described above. However, we now have evidence for other similar reactions occurring in or near the anticodons of other tRNA isoaccepting species. Since these might also be subject to modulation by RNA catabolites and thereby alter mRNA translation, we are attempting to characterize these enzymes.

We have preliminary evidence for a tRNA: hypoxanthine transglycosylase. While isolating tRNA: queuine transglycosylase from rat liver, we observed another enzyme activity eluting at a higher salt concentration from a DEAE cellulose column. This "unknown" enzyme exhibits substrate preferences (ca. 4:1) for hypoxanthine vs guanine and E. coli tRNA vs yeast tRNA, while tRNA: queuine transglycosylase exhibits negligible activity with hypoxanthine and E. coli tRNA. Since E. coli tRNA contains significantly less inosine (the nucleoside of hypoxanthine) than yeast tRNA, we feel the "unknown" enzyme is a tRNA: hypoxanthine transglycosylase, and we are attempting to verify this fact. Inosine is found only in the first (wobble) position of the anticodon of tRNAs

for alanine, arginine, isoleucine, leucine, proline, serine threonine, and valine, although specific cell types may not have all these tRNA species modified to contain inosine. With inosine in the wobble position, mRNA codon recognition is greatly expanded. Therefore, RNA catabolite modulation of this "new" transglycosylase modification reaction could significantly alter phenotypic expression. Further characterization of this and other tRNA modifying enzymes are in progress.

Our studies of chemical carcinogen-induced changes in tRNA metabolism in human cells concentrate on the period subsequent to initiation of carcinogenesis, i.e., the expression or promotion phase. An aspect of the Milo and DiPaolo human cell chemical transformation system that we found especially pertinent to our studies was the inclusion of 8-times the normal concentration of nonessential amino acids to enhance selection for transformed phenotypes after the chemical carcinogen treatment. Based on our proposed model, we felt that the excess amino acids might be required for aminoacylation of altered tRNA isoacceptors; perhaps they are charged less efficiently.

We have discovered that the mouse skin tumor promoter phorbol 12,13-didecanoate (PDD) will promote significant increases in the saturation density of normal human cells cultured in medium containing 8X nonessential amino acids (Trewyn and Gatz, submitted.). The saturation density for the normal control cells was approximately  $4 \times 10^4$  cells/cm<sup>2</sup> at most passages, while the PDD treated cells maintained levels 3 to 4-fold higher for most of the lifetime of the cultures. The specificity of the effect for an active tumor promoter is demonstrated by the fact that the inactive analog 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ -PDD) gave results similar to the untreated controls. A sustained PDD-induced elevation in saturation density of comparable magnitude has been obtained with four different primary cultures in medium supplemented with 8X nonessential amino acids. The greatest phorbol ester-induced increase in the saturation density of diploid human cells previously reported in conventional medium was 50%, and that was transient in nature.

Because aspartic acid and asparagine (2 of the 4 amino acids normally using queuine modified tRNAs) are nonessential amino acids, we next examined combinations of amino acids which include all 4 of those for queuine tRNAs. We discovered an even greater initial increase in the saturation density of normal cells when they were treated with PDD in medium supplemented with 2X aspartic acid, asparagine, histidine, tyrosine (the queuine tRNAs; some of which are hypomodified in transformed cells), and phenylalanine (its tRNA is usually hypomodified for wye base in transformed cells). Cell densities in the PDD treated cultures reached levels 5 to 10-times those in the acetone and 4 $\alpha$ -PDD controls. Similar results have been obtained with 8 different primary cultures in this medium. Although the timing of this transient increase in density has been somewhat variable, all the cultures have exhibited a sustained 2 to 4-fold elevation subsequently, similar to that seen in 8X nonessential amino acids. Removal of PDD from the culture medium results in a return to near-normal saturation density within a few population doublings. The PDD treated cultures remained in exponential growth at cell densities greater than 10-fold higher than the control cultures. Anchorage-independent growth of normal human cells was also promoted by PDD in a dose-dependent manner, with prior subculturing in the presence of PDD being required for maximal colony formation (Trewyn and Gatz, submitted).

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As already discussed, 7-methylguanine induces queuine hypomodification of tRNA and promotes the expression of transformed phenotypes in normal mammalian cells. We now have evidence that phorbol ester tumor promoters elicit similar changes in normal human cells. Preceding the transient increase in saturation density (an in vitro transformed phenotype) promoted by PDD in medium containing 2X aspartic acid, asparagine, histidine, tyrosine, and phenylalanine, there is a transient increase in the queuine hypomodification of cellular tRNA. This data strongly supports our hypothesis that changes in tRNA metabolism are required for the expression of carcinogenesis.

Effects of PDD on carcinogen treated cells are also being studied, although these investigations are in the preliminary stages. When human fibroblasts are grown in the presence of PDD subsequent to carcinogenic insult with  $\beta$ -naphthylamine, anchorage-independent growth and lifespan in culture are enhanced. Presently, we are attempting to optimize these studies of initiation-promotion (using in vitro methods described in the original proposal), since a human cell model of this type has not been described.

Significant changes in DNA synthesis are observed when confluent cultures of human cells are exposed to the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Davakis and Trewyn, in press). TPA exposure results in an initial decrease in thymidine labeling of cellular nucleic acid followed by a sharp increase. An early inhibition of DNA synthesis in cultured mouse cells has been correlated to phorbol ester tumor promotion on mouse skin, so this may be a useful biochemical marker for promoters. At  $10^{-7}$ M concentrations, 4-O-methyl TPA (an inactive analog of TPA), anthralin, norharman, and 7,12-dimethylbenzanthracene do not inhibit or stimulate DNA synthesis in our cultured human cells. These agents also do not increase saturation density of the cells in the 2X amino acid medium as do PDD and TPA. However, the suspected tumor promoters norharman and anthralin do cause a loss of contact inhibition of growth if the human cells have first been exposed to TPA. The significance of these observations to changes in tRNA metabolism are being explored.

d. Publications (appended)

Elliott, M.S. and Trewyn, R.W. Queuine hypomodification of tRNA induced by 7-methylguanine. *Biochem. Biophys. Res. Commun.* 104: 326-332, 1982.

Trewyn, R.W., Elliott, M.S., Glaser, R., and Grever, M.R. Alterations in tRNA metabolism as markers of neoplastic transformation. In: *Biochemical and Biological Markers of Neoplastic Transformation* (P. Chandra, ed.), Plenum Publishing Corp., New York (in press).

Davakis, L.A. and Trewyn, R.W. Evaluating tumor promoter activity in vitro with human diploid fibroblasts. In: *Polynuclear Aromatic Hydrocarbons* (A.J. Dennis and M. Cooke, eds.), (in press).

Trewyn, R.W. and Gatz, H.B. Altered growth properties of normal human cells promoted by phorbol 12,13-didecanoate. *J. Cell. Physiol.* (submitted).

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e. Personnel

Ronald W. Trewyn, Ph.D., Assistant Professor of Physiological Chemistry,  
Principal Investigator.

Holly B. Gatz, Technician (10/1/81 to 8/31/82)

Eric D. Utz, Technician (8/1/82 to present)

Mark S. Elliott, Ph.D. Candidate

Lani A. Davakis, M.S. Candidate

f. Interactions

American Association for Cancer Research, St. Louis, Missouri, April 28 to May  
1, 1982.

Presentation: Putative Tumor Promoter Response in Cultured  
Human Diploid Cells

Published Abstract (appended): Trewyn, R.W., Gatz, H.B., Davakis, L.A.  
and Elliott, M.S. Proc. Amer. Assoc. Cancer Res. 23: 103 1982.

QUEUINE HYPOMODIFICATION OF tRNA INDUCED BY 7-METHYLGUANINE

Mark S. Elliott and Ronald W. Trewyn

Department of Physiological Chemistry, and  
Comprehensive Cancer Center  
The Ohio State University  
Columbus, Ohio 43210

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**SUMMARY:** Transfer RNA isolated from Chinese hamster cells transformed by 7-methylguanine is hypomodified for queuine. 7-Methylguanine rapidly induces queuine hypomodification of tRNA in normal Chinese hamster embryo cells under conditions leading to transformation, and the enzyme catalyzing the queuine modification reaction, tRNA: guanine transglycosylase, is inhibited by 7-methylguanine in vitro.

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Extensive post-transcriptional modification of tRNA generates macromolecules containing a vast array of altered purines and pyrimidines. Queuine (Q-base), a highly modified guanine analog, is one such modification that is found in the first position of the anticodon of tRNA isoacceptors for histidine, tyrosine, aspartic acid, and asparagine (1). Transfer RNA isolated from normal mammalian cells is almost fully modified for Q-base. However, tRNA from tumor tissue or cells transformed in vitro exhibits pronounced hypomodification for Q-base (2). This structural change in the anticodon leads to the appearance in transformed cells of unique tRNA isoaccepting species with altered chromatographic characteristics in vitro (2-4).

The enzymatic modification generating Q-containing tRNA is unusual, in that it involves a direct replacement of Q-base for guanine (5,6). The enzyme responsible for catalyzing this reaction is tRNA: guanine transglycosylase (5,6), and a generalized reaction scheme for the mammalian enzyme is depicted in Figure 1. The enzyme was discovered in rabbit reticulocytes by its ability

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Abbreviations: Q-base, queuine [7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylamino-methyl)-7-deazaguanine]; ChH, normal Chinese hamster embryo cells; ChH-1G, Chinese hamster embryo cells transformed with 1-methylguanine. ChH-7G, Chinese hamster embryo cells transformed with 7-methylguanine.

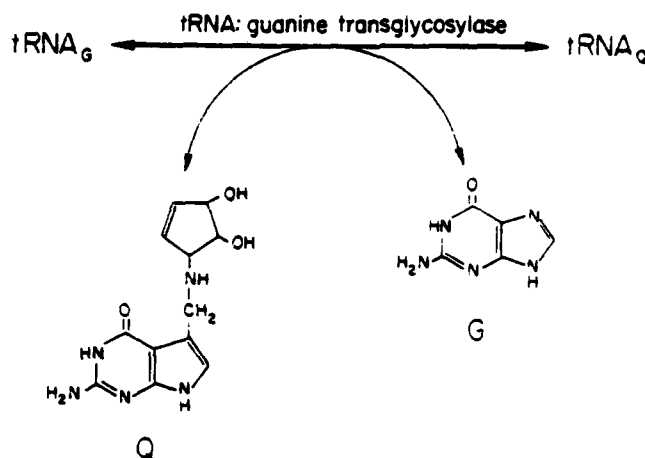


Fig. 1. tRNA: guanine transglycosylase reaction responsible for exchanging Q-base for guanine in the first position of the anticodon of mammalian tRNA for asparagine, aspartic acid, histidine and tyrosine. The abbreviations are: Q, 7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine (queuine); and G, guanine.

to insert radiolabeled guanine into homologous tRNA (7), and with appropriate Q-containing tRNA substrates, the reaction is reversible in vitro (3).

Bacterial tRNA also contains Q-base. However, the tRNA: guanine transglycosylase from *Escherichia coli* utilizes a precursor of Q-base, 7-(aminomethyl)-7-deazaguanine, as the initial substrate, and then the modification is completed at the macromolecular level (9). *E. coli* tRNA: guanine transglycosylase has been characterized extensively (2,9,10), and it has been shown to be inhibited very effectively by 7-methylguanine (10).

It was demonstrated previously that chronic exposure of normal Chinese hamster embryo cells to 7-methylguanine or 1-methylguanine can lead to altered cellular growth properties, including in vitro neoplastic transformation (11-13). How normal components of cellular nucleic acids (7-methylguanine and 1-methylguanine) can influence phenotypic expression when supplied to the cell's culture environment has not been established. However, the reported inhibition of *E. coli* tRNA: guanine transglycosylase by 7-methylguanine led us to investigate whether the methylated purines transforming Chinese hamster cells could inhibit Q-base modification of tRNA. It was surmised that a major structural change (guanine vs Q-base) in the anticodon of specific tRNA species

might play some role in altered gene expression in cells exposed to elevated methylated purine concentrations for prolonged periods.

#### MATERIALS AND METHODS

Establishment and propagation of primary cultures of Chinese hamster embryo (ChH) cells were as previously described except that the culture medium was supplemented with 5% fetal bovine serum (11,12). Establishment of transformed ChH cell lines by chronic exposure to 10  $\mu$ M 1-methylguanine (ChH-1G) and 10  $\mu$ M 7-methylguanine (ChH-7G) was reported previously (11). Fetal bovine serum deficient in Q-base was prepared by treatment with dextran-coated charcoal as described by Katze (3). Proliferating cells were homogenized in hypotonic buffer, and tRNA was isolated as described by Wilkinson and Kerr (14).

Transfer RNA: guanine transglycosylase was isolated from *E. coli* MRE 600 cells (Grain Processing Corp.) as described by Okada and Nishimura (10). However, purification of the enzyme was carried only through the DEAE-cellulose chromatography step, since the preparation was free of RNase activity when the RNase-deficient strain MRE 600 was utilized. The *E. coli* tRNA: guanine transglycosylase was used to assay for Q-hypomodified tRNA as reported by Okada et al. (2). The assay procedure was that of Howes and Farkas (15) with a modified reaction mixture containing: 10  $\mu$ moles Tris-HCl (pH 7.4), 53  $\mu$ moles KCl, 5  $\mu$ moles 2-mercaptoethanol, 1 mmole [8- $^3$ H]guanine (1 Ci/mmole), 0.05 to 0.25 A<sub>260</sub> units tRNA, and 6 units *E. coli* tRNA: guanine transglycosylase in a total volume of 0.6 ml. Transfer RNA was precipitated and collected on glass fiber filters for scintillation counting (15).

Rabbit erythrocyte lysates were prepared as the source of a mammalian tRNA: guanine transglycosylase as described by Howes and Farkas (15). The lysate was centrifuged at 20,000 x g for 20 minutes and 105,000 x g for 90 minutes and desalted on a Sephadex G-25 column. This enzyme preparation was used to assay 1-methylguanine and 7-methylguanine as enzyme inhibitors. The reaction mixture was as described above for Q-hypomodified tRNA using the *E. coli* enzyme except 2.0 A<sub>260</sub> units of Q-deficient yeast tRNA was included as a substrate and the guanine concentration was 1  $\mu$ M. In this case, the reaction was terminated and extracted with phenol as described by Farkas and Singh (7) before precipitating and collecting the tRNA on glass fiber filters.

#### RESULTS AND DISCUSSION

Transfer RNA isolated from transformed ChH-1G and ChH-7G cells was assayed for deficiency of Q-base using the *E. coli* enzyme, and the results are presented in Table 1. The tRNAs from cells transformed by the methylated purines were approximately 5-fold better tRNA: guanine transglycosylase substrates than the tRNA from normal ChH cells. The tRNAs from the transformed cells were also better substrates in the assay than the positive control, yeast tRNA.

To determine whether 1-methylguanine and/or 7-methylguanine can inhibit Q-base modification of cellular tRNA directly, normal ChH cells were exposed to the individual methylated purines for six population doublings. *E. coli* tRNA:

TABLE 1. Queuine-hypomodified tRNA from Chinese hamster cells transformed by methylated purines.

tRNA Source	Guanine Incorporation (pmoles/hr/A <sub>260</sub> unit) <sup>a</sup>
ChH	3.9 ± 0.4
ChH-1G	17.6 ± 1.5 <sup>b</sup>
ChH-7G	19.9 ± 6.8 <sup>b</sup>
Yeast	6.6 ± 1.3
<i>E. coli</i>	<0.2

<sup>a</sup> Mean ± standard deviation from 4 experiments.<sup>b</sup> Significantly different from normal ChH cells;  $P < 0.05$ .

guanine transglycosylase was again used to assay for deficiency of Q-base in the tRNA isolated from the cells, and these results are presented in Figure 2. As can be seen, the short-term exposure of the normal diploid cells to 10  $\mu$ M 7-methylguanine led to the induction of Q-deficient tRNA. However exposure to

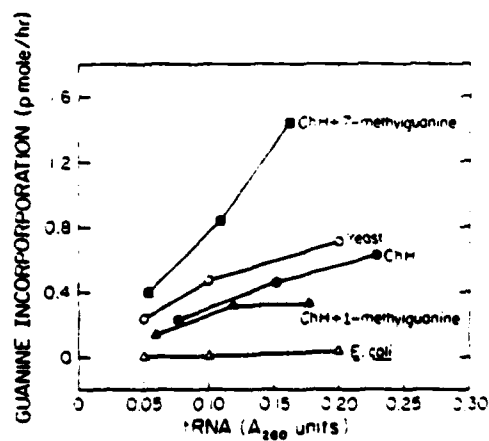


Fig. 2. Hypomodification of ChH tRNA for Q-base induced by 7-methylguanine. Transfer RNA was isolated from ChH cells treated for 6 population doublings with 10  $\mu$ M 7-methylguanine (■), from ChH cells treated for 6 population doublings with 10  $\mu$ M 1-methylguanine (▲), and from matched untreated ChH cells (●). Yeast tRNA (○) and *E. coli* tRNA (△) were used as Q-deficient and Q-sufficient controls respectively. Triplicate reactions were run at each tRNA concentration. See Materials and Methods for additional details.

10  $\mu$ M 1-methylguanine did not lead to Q-hypomodification of tRNA after six population doublings (Figure 2).

It has been reported that mouse cells either cannot synthesize Q-base or cannot synthesize enough for their needs (16), and therefore, cells in culture obtain most of this modified purine from the serum utilized to supplement the growth medium (5,17). To establish whether ChH cells also require exogenous Q-base for proper tRNA maturation, cells were grown for two population doublings in Q-deficient serum after which the tRNA was assayed for Q-hypomodification. The results are presented in Figure 3, and the requirement for exogenous Q-base is clearly evident.

Transfer RNA: guanine transglycosylase from rabbit erythrocytes was used to assess the direct inhibition of tRNA modification in vitro by 1-methylguanine and 7-methylguanine. These results are presented in Figure 4. As can be seen, 10  $\mu$ M 7-methylguanine effectively inhibited the mammalian enzyme, whereas 1-methylguanine was much less effective. These results for the isolated rabbit erythrocyte enzyme are consistent with those for the cultured ChH cells presented in Figure 2. However, it remains to be established whether the induction of Q-hypomodification of ChH tRNA by 7-methylguanine occurs by a direct inhibition of the ChH transglycosylase or by the inhibition of transport of exogenous Q-base. These studies are underway, as are examinations of the kinetics of enzyme inhibition.

The possibility that tRNA: guanine transglycosylase inhibitors are responsible for the Q-hypomodification of tRNA in malignant cells in vivo was suggested by other investigators based on comparable enzyme activities being observed in normal Q-sufficient and malignant Q-deficient tissues (6,18). The nature of these putative inhibitors was not established. However, it is known that malignant tissues contain aberrant tRNA methyltransferases (19), and that cancer patients excrete highly elevated levels of tRNA catabolites; especially methylated derivatives (19,20). Therefore, it is possible that increased endogenous methylated purines (e.g. 7-methylguanine) may be involved in inducing the Q-hypomodification of tRNA associated with neoplasia. This could

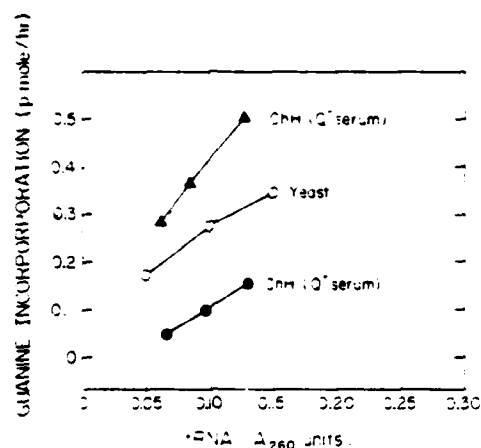


Fig. 3. Hypomodification of O<sub>6</sub>m tRNA for Q-base induced by culturing cells in Q-deficient serum. Transfer RNA was isolated from O<sub>6</sub>m cells grown for 2 population doublings in Q-deficient (Q<sup>-</sup>) serum (▲) or matched Q-sufficient (Q<sup>+</sup>) serum (●). See legend to Figure 2 and Materials and Methods for additional details.

explain how Q-deficient tRNA is generated in normal tissues far from the tumor origin in animals (21). In addition, it offers an explanation for the source of Q-deficient tRNA in the O<sub>6</sub>m-1G cells, since it was demonstrated previously that the tRNA methyltransferase activity in these cells is elevated significantly (11).

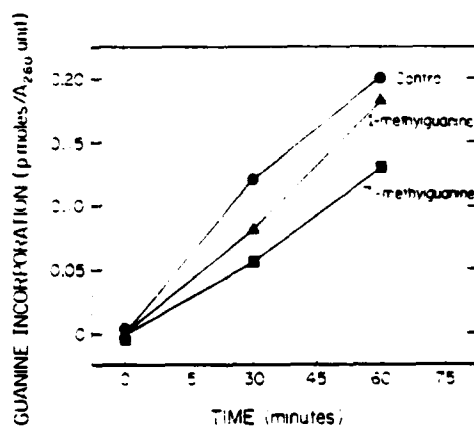


Fig. 4. Inhibition of tRNA: guanine transglycosylase from rabbit erythrocytes by methylated guanines. The assays were carried out as described in the Materials and Methods with the guanine substrate concentration at 1  $\mu$ M. The methylguanines were added at time zero at a final concentration of 10  $\mu$ M. The reactions are: Control (●), plus 1-methylguanine (▲), and plus 7-methylguanine (■).

Studies are underway to determine if inhibition of tRNA modification by 7-methylguanine or other methylated purines excreted at high levels by cancer patients has some fundamental role in the expression of carcinogenesis. A model invoking such a role was proposed previously (22).

#### ACKNOWLEDGMENTS

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## ALTERATIONS IN tRNA METABOLISM AS MARKERS OF NEOPLASTIC TRANSFORMATION

Ronald W. Trewyn, Mark S. Elliott,  
Ronald Glaser, and Michael R. Grever

Department of Physiological Chemistry (R.W.T., M.S.E.)  
Department of Medical Microbiology & Immunology (R.G.)  
Department of Medicine (M.R.G.)  
The Ohio State University  
Columbus, Ohio 43210

### INTRODUCTION

Numerous changes in tRNA modification and catabolism are observed when cells undergo neoplastic transformation. The nature of certain of these changes is well established, while others require further characterization. Enzymes involved in the macromolecular modification of tRNA, the tRNA methyltransferases, exhibit idiosyncratic alterations during neoplastic transformation. These alterations include increases in enzyme specific activity as well as the appearance of different tRNA methyltransferases in the malignant tissue<sup>1,2</sup>. The increased tRNA methyltransferase activity and capacity observed in vitro for the enzymes isolated from transformed cells can also be correlated to increased methylation of specific tRNA isoaccepting species, although not total tRNA, in vivo<sup>3</sup>.

Elevated turnover of tRNA is another characteristic of neoplastic transformation<sup>4,5</sup>, and the increased rate of tRNA catabolism may explain the lack of extensive hypermethylation of total tRNA in malignant cells. Most modified nucleosides in tRNA cannot be salvaged when the macromolecules are degraded, and therefore, they are excreted<sup>4</sup>. Monitoring the elevated excretion of these tRNA catabolites by cancer patients is being investigated to determine the usefulness of these components as biochemical markers for cancer<sup>6-8</sup>.

The enhanced generation of methylated tRNA catabolites by cancer patients may also have a fundamental role in the neoplastic process. Chronic exposure of normal mammalian cells in culture to specific methylated purine RNA catabolites can lead to neoplastic transformation<sup>9,10</sup>. However, the mode of action by which these natural products elicit such a response has not been established.

The appearance of many unique tRNA isoaccepting species is another common feature established for malignant cells<sup>4,11,12</sup>. In some cases, these species appear to differ from their normal counterparts with respect to their macromolecular modifications. Hypomodification for Y-base adjacent to the anticodon in phenylalanine tRNA and for Q-base in the first position of the anticodons for histidine, tyrosine, asparagine, or aspartic acid tRNA's is responsible for the appearance of some of the different isoacceptors in transformed cells<sup>12-14</sup>. Again, these tRNA aberrations offer biochemical markers for neoplastic transformation.

In this report, we examine certain of the alterations in tRNA metabolism associated with neoplasia. Potential interrelationships between the changes in tRNA modification and catabolism are explored, and a role for these aberrations in the expression of carcinogenesis is postulated.

#### MATERIALS AND METHODS

Nucleosides in urine were resolved and quantitated using reversed-phase high performance liquid chromatography<sup>8</sup> following clarification on a boronate column<sup>15</sup>. Quantitation was relative to the creatinine content in random urine specimens<sup>7</sup>.

Establishment and propagation of primary cultures of Chinese hamster embryo cells were as previously described<sup>9,16</sup> except that the culture medium was supplemented with only 5% fetal bovine serum. These cells typically exhibit a finite lifetime in culture of 10 to 12 passages under the conditions employed. The methods for transforming these cells by chronic exposure to selected methylated purines have also been published<sup>9,10</sup>. The concentration of methylated purine utilized was always 10  $\mu$ M.

The assay for Q-deficient tRNA makes use of the enzyme tRNA transglycosylase from Escherichia coli<sup>14</sup>. This enzyme can utilize mammalian tRNA's for histidine, tyrosine, asparagine, and aspartic acid as substrates only if they are hypomodified, i.e., the tRNA's have guanine in the first position of the anticodon instead of Q-base<sup>14</sup>. Transfer RNA from proliferating Chinese hamster cells treated with 10  $\mu$ M 7-methylguanine was isolated utilizing published protocols<sup>17</sup>, and it was evaluated as a substrate for the E. coli enzyme. The assay for Q-hypomodification involves an exchange

reaction with [8-<sup>3</sup>H]guanine. Previously published methods were employed<sup>14,18</sup>. Yeast tRNA is Q-deficient, and therefore, was utilized as a positive control. *E. coli* tRNA is Q-sufficient, so it was used as a negative control. A tRNA transglycosylase from rabbit erythrocytes<sup>19</sup> was used to assess enzyme inhibition by 7-methylguanine.

## RESULTS

### Nucleoside Excretion by Cancer Patients

Urinary nucleoside excretion has been quantitated for patients with nasopharyngeal carcinoma (NPC) and leukemia, and the results

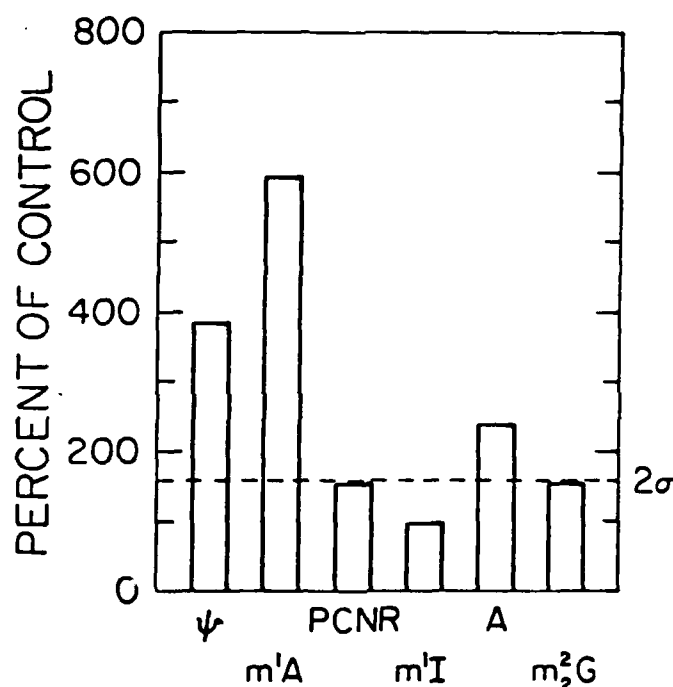


Fig. 1. Excretion of nucleoside markers by a patient at the time of diagnosis of NPC. The results were calculated as nmoles nucleoside/ $\mu$ mole creatinine, and are expressed relative to normal values as percent of control. The dashed line denotes the position of two standard deviations above normal for adenosine, the marker exhibiting the largest relative standard deviation. Excretion levels above the dashed line represent significant ( $P < 0.02$ ) increases for adenosine and highly significant ( $P < 0.01$ ) for the other nucleosides. The abbreviations are:  $\psi$ , pseudouridine; m<sup>1</sup>A, 1-methyladenosine; PCNR, 2-pyridone-5-carboxamide-N'-ribofuranoside; m<sup>1</sup>I, 1-methylinosine; A, adenosine; and m<sup>2</sup>G, N<sup>2</sup>,N<sup>2</sup>-dimethylguanosine.

have been compared to normal excretion levels. The normal excretion values (nmoles nucleoside/ $\mu$ mole creatinine) used for comparison were as follows<sup>8</sup>: pseudouridine, 24.8; 1-methyladenosine, 2.02; 2-pyridone-5-carboxamide-N'-ribofuranoside, 1.14; 1-methylinosine, 0.96; 1-methylguanosine, 0.70; adenosine, 0.23; and N<sup>2</sup>,N<sup>2</sup>-dimethylguanosine, 1.05. The greatest relative standard deviation for the controls was 30.4% for adenosine.

The relative nucleoside excretion pattern for a Caucasian NPC patient at the time of diagnosis is presented in Fig. 1. At that time, the excretion levels of pseudouridine and 1-methyladenosine were elevated 4-fold and 6-fold respectively. Cells from tumor tissue biopsies contained the Epstein-Barr virus (EBV) genome (12.5 equivalents/cell)<sup>20</sup>, and the patient's serum contained very high levels of antibodies to an EBV-specific DNase (10.2 units neutralized/ml serum).

The nucleoside excretion pattern for an individual at the time of diagnosis of acute myelogenous leukemia (AML) is shown in Fig. 2. In this case, 1-methylinosine was the primary marker with an increase of greater than 14-fold. Four of the other nucleosides (pseudouridine, 2-pyridone-5-carboxamide-N'-ribofuranoside, 1-methylguanosine, and N<sup>2</sup>,N<sup>2</sup>-dimethylguanosine) were elevated

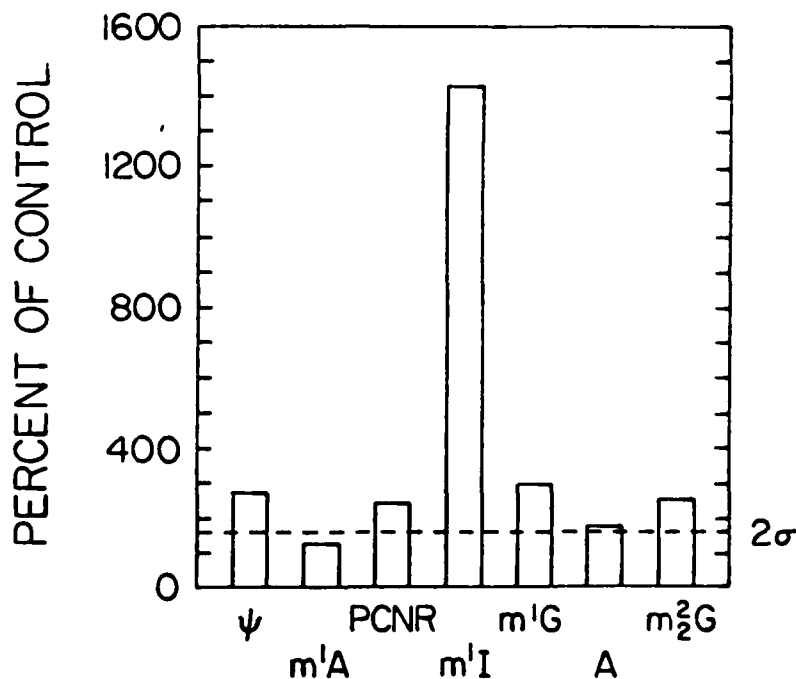


Fig. 2. Excretion of nucleoside markers by a patient at the time of diagnosis of AML. See legend to Fig. 1 for details. The additional abbreviation is:  $m^1G$ , 1-methylguanosine.

approximately 3-fold. This patient also had significantly elevated adenosine deaminase levels in his peripheral blood cells (21.0 units/ $10^6$  cells) compared to normal (8.4 units/ $10^6$  cells).

#### Cell Transformation by Methylated Purines

The significant increase in the excretion of modified RNA catabolites by cancer patients led to the examination of the response of normal mammalian cells to these components<sup>9,10</sup>. Certain methylated purines were found to transform Chinese hamster embryo cells in vitro, with neoplastic transformation being demonstrated in some cases<sup>10</sup>. A summary of methylated purines evaluated and those transforming the cells for proliferative capacity (finite to continuous lifetime in culture) can be seen in Table 1. Certain of the RNA catabolites (1-methylguanine and 7-methylguanine) greatly enhance the generation of continuous cell lines, while others do not. Other naturally occurring methylated purines (7-methylxanthine and 1,3,7-trimethylxanthine) are also quite effective in transforming the cells.

The expression of various transformed phenotypes appearing during continuous exposure to the methylated purines can be reversed by removal of the methylated purine. An example showing increased saturation density of a 7-methylxanthine-transformed cell line is presented in Fig. 3. Removal of 7-methylxanthine at passage level 15, a passage level exceeding the normal number of passages obtainable before senescence and cell death, resulted in a significant decrease in the cell density of subsequent passage levels. In 3 of 4 independent transformation experiments with 7-methylxanthine, the cultures went through such a "crisis"

Table 1. Methylated Purines Generating Continuous Chinese Hamster Cell Lines

Methylated Purine	Continuous/Treated
None (Control)	2/30
Guanine (Control)	0/2
1-Methyladenine	0/2
1-Methylguanine	15/16
3-Methylguanine	0/2
7-Methylguanine	11/12
1-Methylhypoxanthine	0/2
1-Methylxanthine	1/4
3-Methylxanthine	0/2
7-Methylxanthine	4/4
1,3,7-Trimethylxanthine	4/6

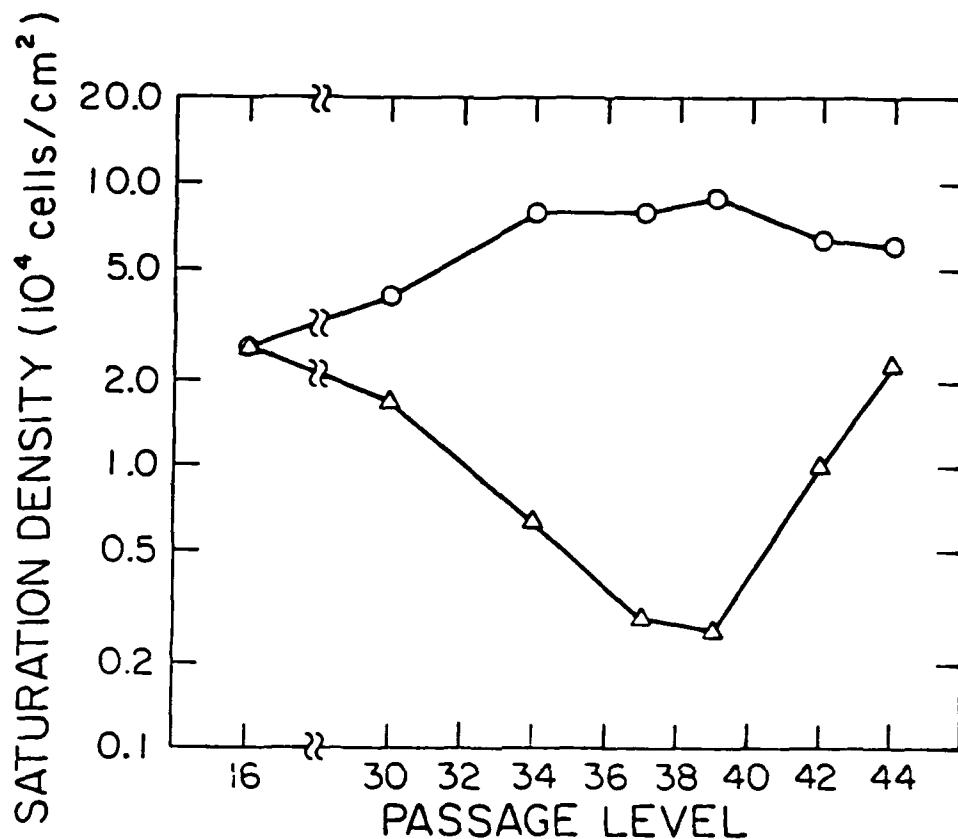


Fig. 3. Saturation densities for a 7-methylxanthine-transformed Chinese hamster cell line. The cells had been treated continuously with 10  $\mu$ M 7-methylxanthine since the first passage of the primary culture. When the transformed, "continuous" cell line was subcultured at passage 15, duplicate cultures were maintained thereafter in the presence (o) or absence ( $\Delta$ ) of 7-methylxanthine. Saturation densities were determined after confluent cultures were split 1:4 and allowed to grow for 7 days. Duplicate cultures were trypsinized, and cells were counted with a hemacytometer.

period after removal of the methylated purine. With a 1,3,7-trimethylxanthine-transformed cell line, the cloning efficiency in soft agar decreased 6-fold after removal of the methylxanthine (unpublished observation), and tumorigenicity in nude mice was reversed by removal of 1-methylguanine from a corresponding cell line<sup>21</sup>. In the latter case, there was no change in the cloning efficiency in soft agar or any other *in vitro* characteristic related to transformation.

### Q-Hypomodification of Cellular tRNA

The enzyme tRNA transglycosylase from mammalian sources catalyzes the reaction depicted in Fig. 4. Transfer RNA isolated from normal cells is mainly in the Q-modified form, while tRNA from transformed cells is Q-deficient<sup>14</sup>. The possibility that 7-methylguanine, a structural analog of Q-base, might inhibit Q-modification of tRNA was examined by treating normal Chinese hamster cells with 10  $\mu$ M 7-methylguanine; the same concentration and conditions used for transformation. Transfer RNA was isolated from treated and untreated normal cells after 6 population doublings and assayed for Q-deficiency. Transfer RNA was also isolated from cells treated for 4 population doublings followed by no treatment for 2 more doublings to assess reversibility of any 7-methylguanine-induced Q-hypomodification. As can be seen in Table 2, 7-methylguanine induced Q-hypomodification of tRNA in the cells, and the Q-deficiency was reversible.

A tRNA transglycosylase isolated from rabbit erythrocytes was also shown to be inhibited by 10  $\mu$ M 7-methylguanine in vitro. In 4 separate experiments with Q-deficient yeast tRNA, the percent inhibition obtained was  $60.1 \pm 7.6$  (mean  $\pm$  standard deviation) when the guanine substrate concentration was 1  $\mu$ M.

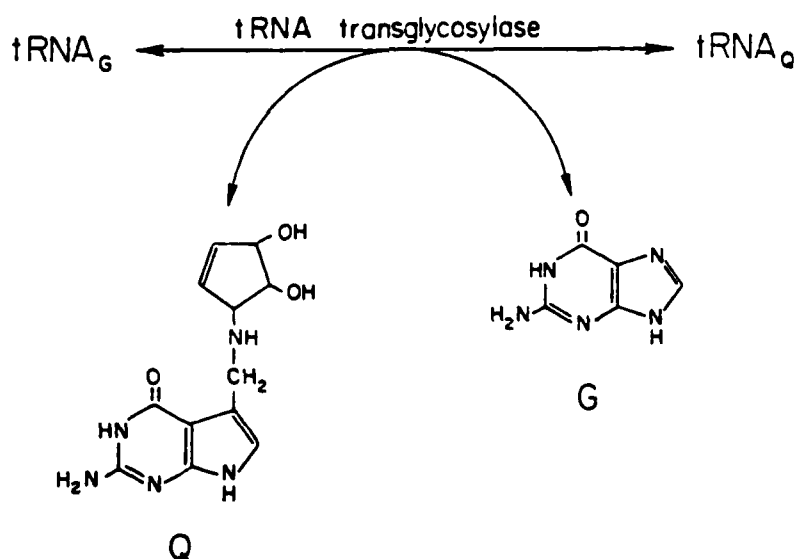


Fig. 4. tRNA transglycosylase reaction responsible for exchanging Q-base for guanine in the first position of the anticodon of tRNA's for tyrosine, histidine, asparagine, and aspartic acid. The abbreviations are: Q, 7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine; and G, guanine.

Table 2. Q-Hypomodification of tRNA in Chinese Hamster Cells Induced 7-Methylguanine

tRNA Source	Guanine Incorporation (pmoles/hr/A <sub>260</sub> unit)
Chinese hamster cells	1.86
Plus 7-methylguanine	3.46
Minus 7-methylguanine	2.09
<u>Escherichia coli</u>	<0.2
Yeast	6.58

#### DISCUSSION

The potential value of modified nucleosides as biochemical markers for cancer can be seen in Fig. 1 and Fig. 2. Even at the time of cancer diagnosis, nucleoside excretion was elevated significantly for the NPC patient (Fig. 1), and this correlated with high serum antibodies to EBV antigens including antibody to the EBV DNase, a marker for NPC<sup>22</sup>. The AML patient exhibited even higher nucleoside excretion levels at the time of diagnosis (Fig. 2), although the pattern of elevated excretion was different. Unique excretion patterns may offer additional means to characterize specific cancers. The patient with AML also exhibited elevated peripheral blood cell adenosine deaminase activity, another potential biological marker for leukemia. The clinical value of monitoring various markers for leukemia and NPC is being assessed for both diagnostic and prognostic purposes.

The early increases in tRNA catabolism associated with neoplasia led to an examination of the potential role of the catabolites in neoplastic transformation. The discovery that chronic exposure to some, but not all, methylated purines derived from cellular RNA can transform normal diploid cells was quite perplexing<sup>9,10</sup>. However, it appears that the methylated purines may influence the expression of various transformed phenotypes. Removal of the transforming methylated purine at the appropriate time can result in reversal of expression of various transformed phenotypes, e.g., increased proliferative capacity (Fig. 3), anchorage independent growth, and tumorigenicity<sup>21</sup>. All transformed phenotypes are not reversed by removing the methylated



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purine from a particular culture. However, it was demonstrated previously that the methylated purine-transformed cells exhibit elevated tRNA methyltransferase activity<sup>9</sup>, and therefore, the endogenous methylated purine level may negate the need for an exogenous source.

The results obtained with the methylated purines suggested similarities to dedifferentiation associated with carcinogenesis. Since dedifferentiation reportedly involves changes in gene regulation at the post-transcriptional level<sup>23</sup>, we have attempted to identify cellular targets for the methylated purines that might alter phenotypic expression by similar means. A proposed target was tRNA transglycosylase, since the enzyme from *E. coli* is inhibited by the methylated purine 7-methylguanine<sup>18</sup>. It was presumed that a major structural change (guanine vs Q-base) in transformed tRNA's for histidine, tyrosine, asparagine, and/or aspartic acid generated by inhibiting the transglycosylase might allow the altered tRNA isoaccepting species to translate disparate mRNA's more efficiently. As we have now found, 7-methylguanine does inhibit tRNA transglycosylase from a mammalian source, and it induces Q-hypomodification of cellular tRNA (Table 2) under conditions leading to the expression of transformation.

The question of whether Q-deficient tRNA's actually have some role in the expression of transformed phenotypes remains to be answered. However, it has been reported that reversing tRNA Q-deficiency in tumor cells by administration of purified Q-base was associated with diminution of tumor cell growth *in vivo*<sup>24</sup>.

The numerous alterations in tRNA metabolism associated with neoplasia have led us to devise a scheme by which they may interrelate in the expression of carcinogenesis, and the proposed sequence of events is presented in Fig. 5. The induction (initiation) of carcinogenesis could be by any means. The subsequent events are then predicted to have cause and effect relationships, i.e., each change depicted would occur in order and be caused by the previous change. Therefore, soon after the induction event there would be an increase in tRNA methyltransferase activity which would result in an increase in methylated RNA catabolites. The higher endogenous levels of methylated purines would then modulate tRNA modification by inhibiting tRNA transglycosylase. The methylated purines might also modulate tRNA modification by acting as an alternate substrate for the transglycosylase or by other, as yet unidentified, means. Both the modulation and methylation steps would be involved in generating altered tRNA isoaccepting species, and these species might allow the translation of different mRNA's that are not translated efficiently by the normal tRNA population. It is then assumed that some of these translation products would be onco-developmental proteins. If

## EXPRESSION OF CARCINOGENESIS

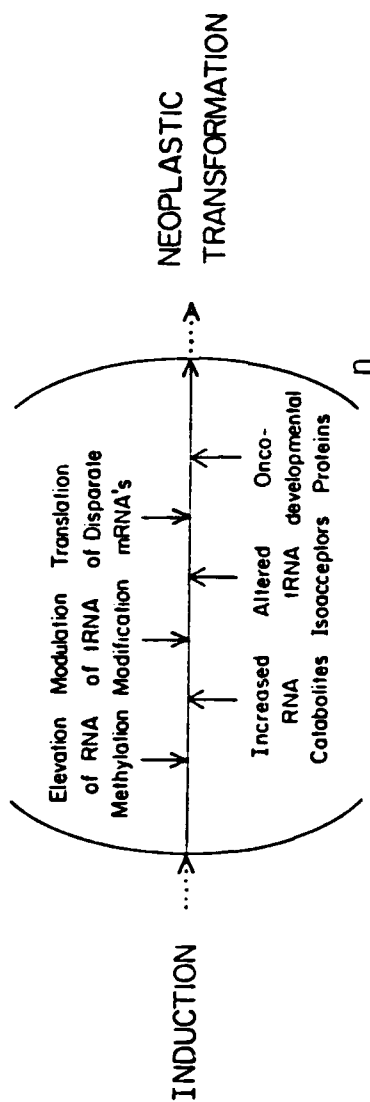


Fig. 5. Proposed model for the role of altered tRNA metabolism in the expression of carcinogenesis. Each event in the sequence is predicted to influence subsequent events, and the last may reinitiate the first.

any of these proteins were tRNA methyltransferases, and it is known that enzymes with different specificities appear<sup>1</sup>, the cycle would repeat at a more aberrant level. By this means, the cycle could continue to generate accruing phenotypic alterations until neoplastic transformation is attained.

The proposed model offers an explanation for the general staging process of carcinogenesis. It also allows interpretation of the phenotypic reversibility phenomenon demonstrated for the exogenous methylated purines. A step back to the previous cycle might be possible by such a withdrawal, but the increased generation of endogenous methylated purines would block any further phenotypic reversion.

Certain of the individual points outlined in Fig. 5 have been proposed by other investigators to have a role in neoplastic transformation. However, linking the increased RNA catabolites to the expression of transformed phenotypes as well as the induction of tRNA hypomodification allowed us to formulate the comprehensive scheme presented. The hypothesis is being tested using a variety of model systems, and the similarities to promotion of neoplastic transformation are being studied. The numerous biochemical markers involved should greatly facilitate these investigations.

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EVALUATING TUMOR PROMOTER ACTIVITY IN VITRO WITH HUMAN

DIPLOID FIBROBLASTS

LANI ANN DAVAKIS, RONALD W. TREWYN\*

Department of Physiological Chemistry, The Ohio State University, 333 West Tenth Avenue, Columbus, Ohio 43210

INTRODUCTION

Carcinogenesis is a long-term process involving the interaction of many endogenous and exogenous factors. The role of chemical pollutants in this process is complex and not well understood in most cases. The study of chemically-induced cancer can be subdivided into the stages of initiation and promotion. Classical initiation/promotion experimental protocols involve the application on mouse skin of sub-carcinogenic doses of an initiator [e.g. the polynuclear aromatic hydrocarbon (PAH) benzo(a)pyrene] followed by repeated applications of a non-carcinogenic tumor promoter [e.g. the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)]. In general, this initiation/promotion scheme in vivo can also be achieved in vitro with cultured rodent cells. The means by which phorbol esters and other agents promote carcinogenesis remains a subject of conjecture. Even in the absence of chemical carcinogen initiation in vitro, tumor promoters are able to induce reversible phenotypic changes in rodent cells that are characteristic of in vitro transformation; a phenomenon described as mimicry of transformation (1).

Although the rodent cell systems offer useful models for many studies of initiation/promotion and mimicry of transformation, the development of a human cell model would be highly desirable for the study of interactions of exogenous chemical agents in human cancer. Milo et al. (2) have demonstrated neoplastic transformation of normal human fibroblasts in vitro subsequent to treatment with complete chemical carcinogens, i.e., carcinogens that do not require promotion. However, classical initiation/promotion has not been achieved with human cells in vitro due to the lack of an appropriate model for the promotion stage. Diamond et al. (3) did report growth stimulation of normal human fibroblasts treated with TPA, but the transient 50% increase in the saturation density of TPA treated cells was attributed to a decrease in cell size induced by TPA. Few other studies on normal human cells in culture have described effects of tumor promoters that could be equated to mimicry of transformation. Tumor promoters alone do induce changes in the growth

## TUMOR PROMOTER RESPONSE IN HUMAN CELLS

characteristics of mutant human fibroblasts (4), and they also influence growth, differentiation, and DNA synthesis in human cancer cells (5,6,7).

Because few phorbol ester-induced effects on normal human cells have been demonstrated and those that have are not of the magnitude reported for rodent cells, it might be concluded that promotion of carcinogenesis is not relevant in man. Epidemiological studies suggest, however, that promotion is an important consideration. Therefore, we are developing a normal human cell culture system responsive to tumor promoters; one which should allow the study of mimicry of transformation as well as two-stage (initiation/promotion) carcinogenesis.

### MATERIALS AND METHODS

#### Chemicals

The potential promoting compounds examined in this study included the phorbol esters TPA and 4-O-methyl-TPA (P.L. Biochemicals Inc., Milwaukee, Wisconsin). The former is the most potent tumor promoter on mouse skin, while the latter is an inactive structural analog (8). Mezerein, another plant diterpene and much weaker promoter than TPA on mouse skin (9), was obtained from Dr. L. David Tomei, Comprehensive Cancer Center, The Ohio State University. Other non-phorbol esters evaluated were anthralin, (1,8-dihydroxy-9-anthrone), 7,12-dimethylbenz(a)anthracene (7,12-DMBA) (both courtesy of Dr. George E. Milo, Department of Physiological Chemistry, The Ohio State University), and norharman, (9H-pyrido-[3,4-b]indole), (Sigma Chemical Co., St. Louis, Missouri). Anthralin is also a tumor promoter on mouse skin (10), while norharman, a component of tobacco smoke, is a co-mutagen (11). The PAH 7,12-DMBA is a complete carcinogen in rodent systems, but is inactive with normal human cells in vitro (12).

#### Cell Culture

Primary cultures of normal human fibroblasts were prepared from neonatal foreskin as described by Riegner et al. (13). The cells were grown in Eagle's minimal essential medium (GIBCO, Grand Island, New York) supplemented with 25 mM HEPES (pH 7.2), 0.2% sodium bicarbonate, 1.0 mM sodium pyruvate, gentomycin (5 µg/ml), 2X vitamins, 1X nonessential amino acids, and 2X aspartic acid, asparagine, histidine,

## TUMOR PROMOTER RESPONSE IN HUMAN CELLS

phenylalanine, and tyrosine. These modifications were found to enhance changes in phenotypic expression promoted by phorbol esters (manuscript in preparation), and the medium is referred to hereafter as promotion medium. The medium was also supplemented with either 20% fetal bovine serum (Reheis Chemical Co., Phoenix, Arizona, or Sterile Systems, Logan, Utah) or 20% bovine serum (Sterile Systems) for establishing primary cultures. At the first passage of the primary culture, the cells were split at a ratio of 1:4 into 25 cm<sup>2</sup> flasks, and the serum content of the medium was reduced to 10%. Once a week the cells were subcultured at a ratio of 1:10 and counted with a hemacytometer to determine saturation densities. Duplicate cultures were maintained for each treatment, and the cultures were fed with fresh promotion medium three days following subculture.

Each of the potential promoting agents was dissolved in acetone and added to promotion medium at a final concentration of 10<sup>-7</sup>M and 0.01% acetone. For dose-response studies, promotion medium containing 10<sup>-6</sup>M TPA and 0.1% acetone was prepared, from which serial dilutions were made. Control cultures were treated with the 0.01% or 0.1% acetone. Treatment of the cells with agents of interest was started at the first passage of the primary culture and continued throughout all subcultures.

### DNA Synthesis

The system that we utilized to determine DNA synthesis is a modification of a procedure originated by Ball et al. (14) that allows us to grow, treat, and process human fibroblasts in glass scintillation vials. A primary culture of human fibroblasts was trypsinized and counted. Cultures were seeded by pipeting 2.0 ml aliquots of a suspension (0.25 X 10<sup>5</sup> cells/ml) into a series of sterile vials. When plated into the vials, promotion medium plus 20% BS was used. The cells were allowed to establish five days at which time they were confluent. The experiment was started by the addition of 10<sup>-7</sup> M promoter and 0.01% acetone to the quiescent culture. Acetone alone (0.01%) was added to control cultures. To measure DNA synthesis, [<sup>3</sup>H]thymidine (<sup>3</sup>H-TdR) (Sp. act. 5 Ci/mmole) was added to each vial (0.5 µCi/vial) at various intervals. The cells were pulse labeled for 90 minutes and the medium was decanted.

Precursor incorporation was stopped by the immediate addition of 10 ml of ice-cold saline. The cells were gently rinsed 2 times. One ml of 1.5% (v/v) perchloric acid (PCA)



## TUMOR PROMOTER RESPONSE IN HUMAN CELLS

was applied to remove any unincorporated radioactive precursor and fix the cells to the surface of the vial. The acid was removed and counted to monitor any changes in the thymidine pool caused by exposure to promoter. Two further washes with 10 ml of 1.5% PCA were then applied. The vials were rinsed with 10 ml of 95% ethanol and inverted to dry. Finally, one ml of 5% PCA (v/v) was added to each vial and the samples were heated at 80°C for 40 minutes to hydrolyze nucleic acids. After cooling, scintillation fluid was added to each vial and the samples were counted.

### RESULTS

#### Saturation Density

Significant changes in saturation density were observed when the cultured human fibroblasts were exposed continuously to TPA. The cells exhibited a loss of sensitivity to contact inhibition with an extreme degree of overgrowth. Figure 1 shows the saturation density dose response for cells treated with TPA. The saturation density for control cells was approximately 50,000 cells/cm<sup>2</sup> at all passages. Treatment with 10<sup>-8</sup>M TPA gave the largest increase in saturation density above control values (up to 5-fold) in the experiment depicted. TPA at a concentration of 10<sup>-7</sup>M was effective to a lesser degree, followed by 10<sup>-6</sup> and 10<sup>-9</sup>M TPA which produced nearly equal increases. A marked elevation in saturation density was observed for 10 independent primary cultures treated with 10<sup>-7</sup>M TPA, with the maximum treatment values varying from 120,000 to 280,000 cells/cm<sup>2</sup>. Substituting bovine serum for fetal bovine serum in the promotion medium had no significant effect on the TPA-induced response. Removal of TPA from the culture medium at any passage resulted in a return to near the control saturation density by the next passage.

At a concentration of 10<sup>-7</sup>M, continuous treatment with 4-O-methyl-TPA, anthralin, norharman, or 7,12-DMBA had no effect on the saturation density of diploid human fibroblasts, i.e., the values were indistinguishable from the controls. However, when 10<sup>-7</sup>M norharman was added to cells already being treated with 10<sup>-7</sup>M TPA, a significant response was obtained (Figure 2). The norharman-induced increase was 2-fold greater than that induced by TPA alone, and it was transient in nature. It has not been possible to obtain similar effects when TPA and norharman were added concurrently at the first passage of the primary culture. In

# TUMOR PROMOTER RESPONSE IN HUMAN CELLS

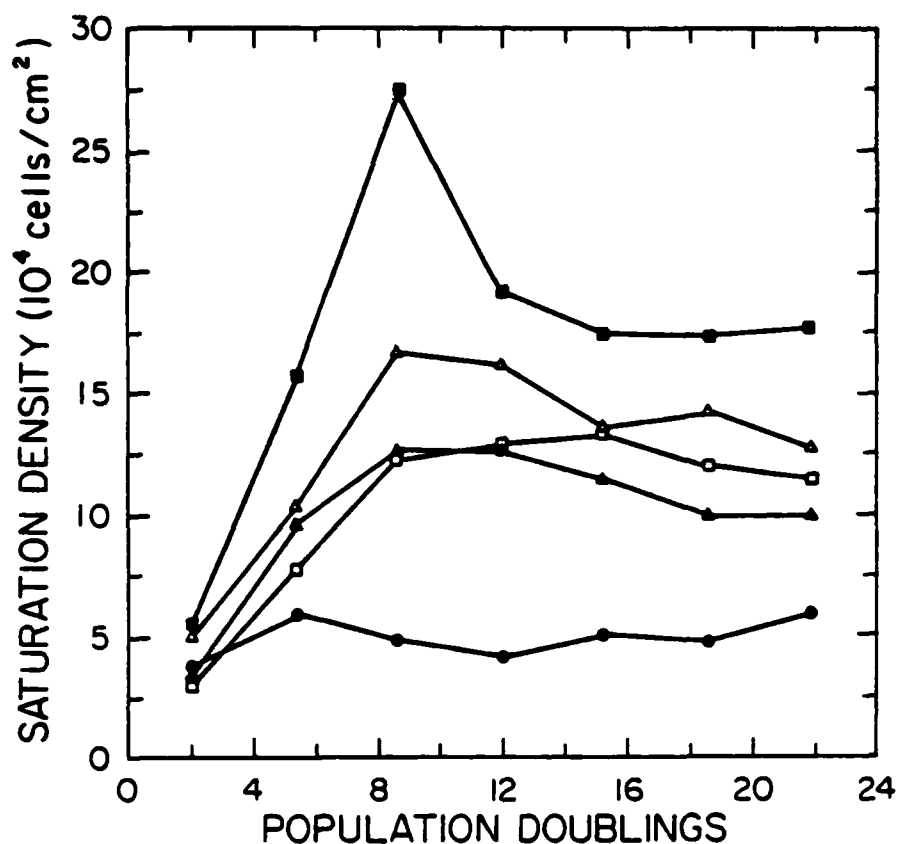
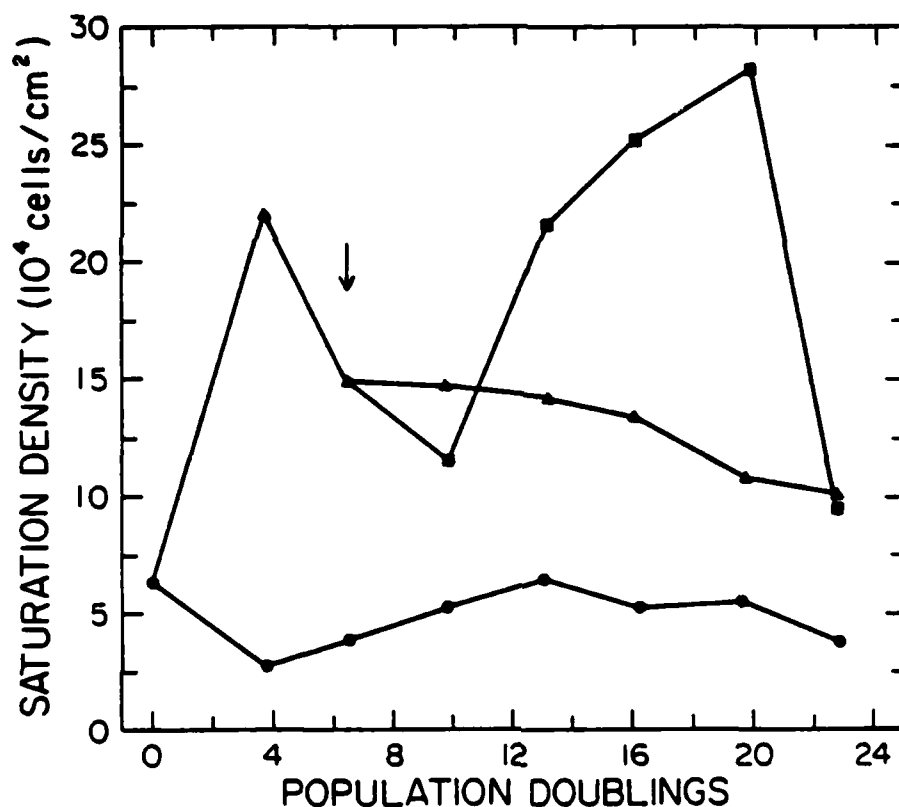


FIGURE 1. TPA dose-response curve. Human fibroblasts treated from passage one with TPA. The concentrations utilized were  $10^{-6}\text{M}$  (▲),  $10^{-7}\text{M}$  (△),  $10^{-8}\text{M}$  (■), and  $10^{-9}\text{M}$  (□). Control cells (●) were treated with 0.1% acetone.

a repeat of the experiment depicted in Figure 2, removal of norharman at any time after the synergistic increase in saturation density, resulted in a return to the TPA density by the subsequent passage.

Anthralin at a concentration of  $10^{-7}\text{M}$  was also found to elevate the saturation density attainable in concert with  $10^{-7}\text{M}$  TPA (Figure 3). As described earlier,  $10^{-7}\text{M}$  anthralin alone was without effect. As shown in Figure 3,  $10^{-7}\text{M}$  mezerein also promoted an increase in growth beyond

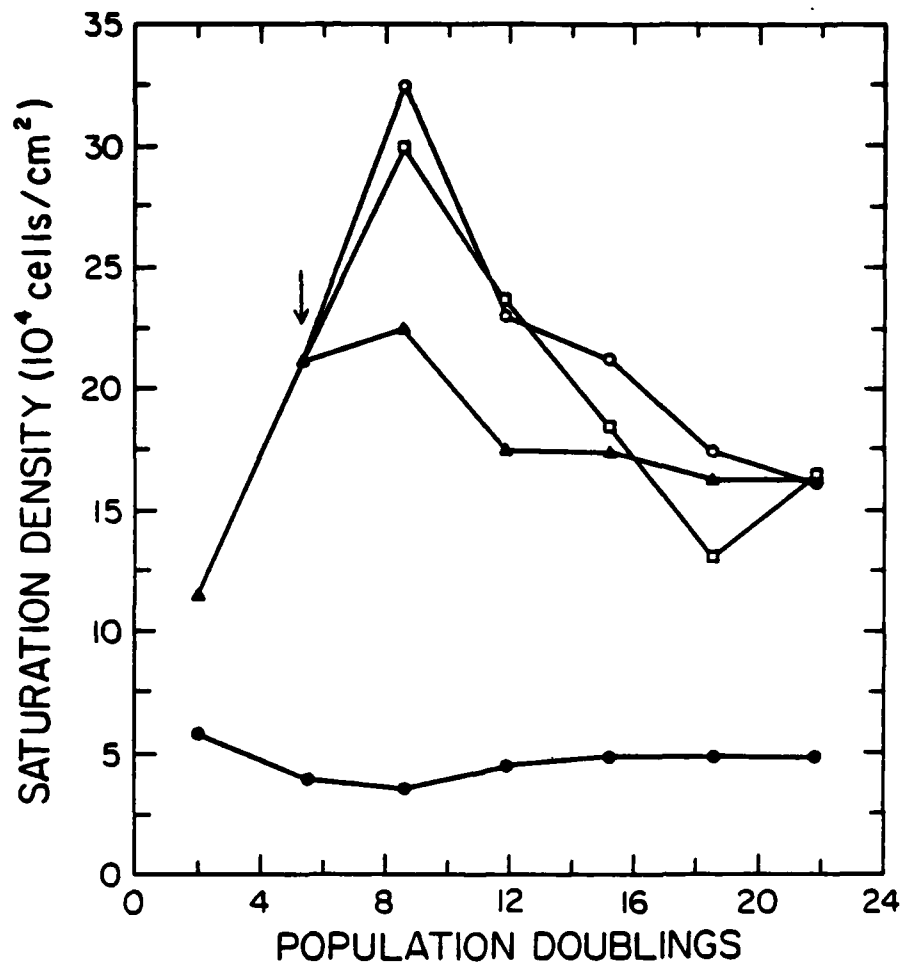
# TUMOR PROMOTER RESPONSE IN HUMAN CELLS



**FIGURE 2.** Effects of TPA plus norharman on saturation density. Cells were exposed continuously to  $10^{-7}$ M TPA (Δ) and after two passages to  $10^{-7}$ M norharman (■). Control cells (●) were treated with 0.01% acetone. The arrow indicates the point at which norharman was added to duplicate cultures.

that obtained with  $10^{-7}$ M TPA. However, continuous treatment with  $10^{-7}$ M mezerein alone causes a reversible 2 to 3-fold increase in the saturation density of the normal human cells, so the effects of TPA plus mezerein may be additive.

# TUMOR PROMOTER RESPONSE IN HUMAN CELLS



**FIGURE 3.** Effects of TPA plus anthralin or mezerein on saturation density. Addition of  $10^{-7}$ M anthralin (□), or  $10^{-7}$ M mezerein (○) to promotion medium of cells already exposed to  $10^{-7}$ M TPA (▲) for three passages. Control cells (●) were treated with 0.01% acetone. The arrow indicates the point at which anthralin and mezerein were added to duplicate cultures.

## TUMOR PROMOTER RESPONSE IN HUMAN CELLS

### DNA Synthesis

Changes in DNA synthesis were also observed with human skin fibroblasts treated with TPA (Figure 4). There was an initial inhibition of  $^3\text{H}$ -TdR incorporation followed by a large stimulation of incorporation. TPA at a concentration of  $10^{-7}\text{M}$  (the concentration depicted) gave the greatest and most prolonged inhibition of DNA synthesis, followed by  $10^{-8}$  and  $10^{-9}\text{M}$ . The DNA stimulatory response was nearly equal for  $10^{-7}$  and  $10^{-8}\text{M}$  TPA and was undetected for  $10^{-9}\text{M}$  TPA. However, the TPA-induced stimulatory (mitogenic) response was less reproducible and more influenced by culture conditions than the inhibitory response. Treatment of human diploid fibroblasts with  $10^{-7}\text{M}$  4-O-methyl-TPA, norharman, anthralin, or 7,12-DMBA had no effect on DNA synthesis. Treatment with norharman or anthralin in conjunction with TPA also had no effect on the inhibition or stimulation of DNA synthesis caused by TPA. Mezerein at a concentration of  $10^{-7}\text{M}$  inhibited DNA synthesis in a transient manner, similar to TPA.

### DISCUSSION

It was established in this investigation that under appropriate culture conditions continuous exposure to TPA or mezerein significantly alters phenotypic and biochemical properties of low passage human diploid fibroblasts. An acute TPA-induced mitogenic response similar to that depicted in Figure 4 has been demonstrated previously with normal human cells (15), but reported phenotypic changes for these cells are minimal (3). The 50% increase in saturation density induced by TPA in the previous studies was attributed to a somewhat smaller cell size (3). Even then, a 50% increase above the normal cell density for human cells of 40,000 to 50,000 cells/cm<sup>2</sup> would yield only 60,000 to 75,000 cells/cm<sup>2</sup>. That is a hardly comparable to levels up to 280,000 cells/cm<sup>2</sup> demonstrated under the conditions prescribed in this investigation. Although a significant elevation in saturation density was induced by TPA, some variability was observed in the magnitude and timing of the response. In addition, the secondary response to norharman, anthralin, and mezerein in TPA treated cultures was greatest when the cells were highly responsive to TPA. This variability suggests differences in promoter sensitivity among primary human skin cell cultures; an aspect currently being explored.

# TUMOR PROMOTER RESPONSE IN HUMAN CELLS

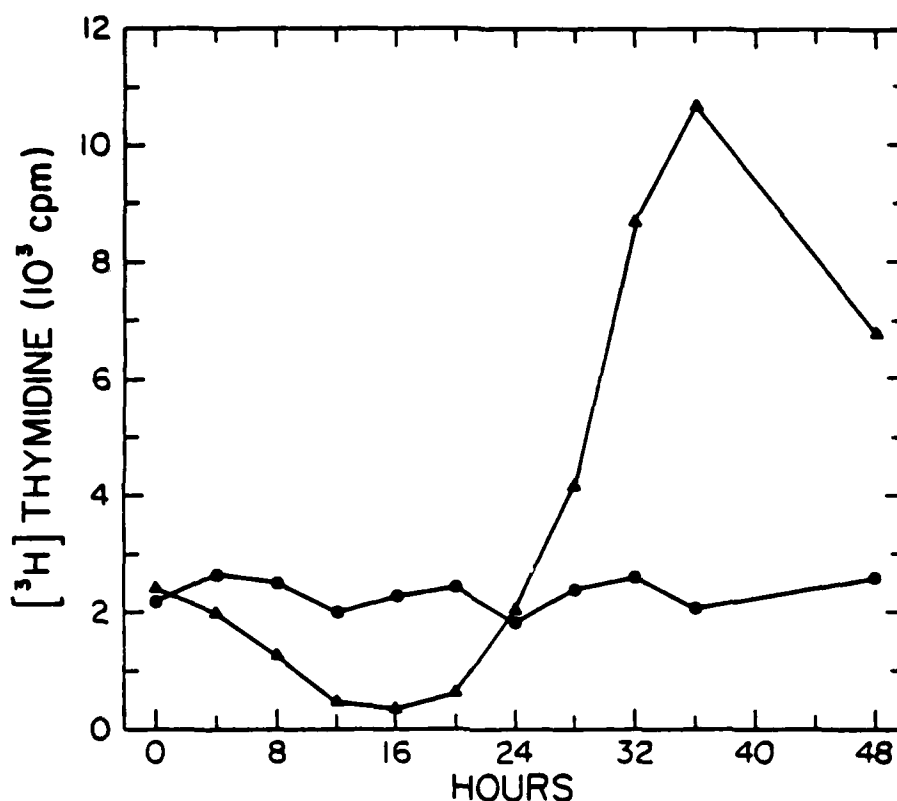


FIGURE 4. Effects of TPA on DNA synthesis. Primary cultures of normal human fibroblasts were seeded and grown in glass scintillation vials (Materials and Methods). Five days later the confluent, quiescent cultures were treated with  $10^{-7}$ M TPA (▲) or 0.01% acetone (●) at time zero. At 4 hour intervals the cells were pulse labeled with  $^3\text{H}$ -TdR.

The only two agents that were able to induce mimicry of transformation by themselves, TPA and mezerein, were also the only two that inhibited DNA synthesis immediately after exposure. Peterson *et al.* (16) suggested that the transient inhibition of DNA synthesis in cultured mouse fibroblasts correlated with promoter activity of various phorbol esters; an observation in agreement with our human fibroblast results. Specificity is indicated by the fact that 4-O-methyl-TPA, anthralin, norharman, or 7,12-DMBA alone neither inhibited DNA synthesis nor promoted a loss of

## TUMOR PROMOTER RESPONSE IN HUMAN CELLS

contact inhibition of growth. Therefore, evaluating agents for their ability to inhibit DNA synthesis in human diploid fibroblasts following acute exposures may offer a rapid means to identify "complete promoters" for these cells.

The effects of norharman and anthralin in conjunction with TPA are more complex. Apparently these compounds are "incomplete promoters" or "co-promoters" for inducing mimicry of transformation of normal human cells. The promotion of mouse skin tumors was reported by Slaga *et al.* (17) and Furstenberger *et al.* (18) to be at least a two-stage process, with some agents functioning as second stage promoters following TPA exposure. Our *in vitro* human cell results are consistent with anthralin and norharman being second stage promoters, but other possibilities have not been totally ruled out. Mezerein may function both as a weak complete promoter and a second stage promoter.

The biochemical and phenotypic changes induced in human diploid fibroblasts by putative tumor promoters may aid in establishing agents which are relevant to human carcinogenesis. In addition to further investigations of mimicry of transformation, conditions are being defined to examine two-stage carcinogenesis with the human cells *in vitro*. By this means we hope to better understand the role of exogenous chemical agents in cancer.

### ACKNOWLEDGEMENTS

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ALTERED GROWTH PROPERTIES OF NORMAL HUMAN CELLS  
PROMOTED BY PHORBOL 12,13-DIDECANOATE

RONALD W. TREWYN AND HOLLY B. GATZ

Department of Physiological Chemistry, and  
Comprehensive Cancer Center  
The Ohio State University  
Columbus, Ohio 43210

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Address all correspondence to: Dr. Ronald W. Trewyn, Department of Physiological Chemistry, The Ohio State University, 1645 Neil Avenue, Columbus, Ohio 43210. Telephone: (614) 422-2546.

Running head: Altered growth of human cells promoted by PDD.

Total text figures = 5.

ABSTRACT The active tumor promoter phorbol 12,13-didecanoate (PDD) significantly alters the growth properties of early passage normal human skin cells in vitro in culture medium supplemented with elevated concentrations of selected amino acids. Continuous treatment of cells with  $10^{-7}$  or  $10^{-8}$ M PDD results in an initial 5 to 10-fold increase in saturation density and/or a long-term 2 to 4-fold increase. Removal of PDD from the culture medium results in a rapid return to near normal saturation density. The PDD treated cultures remain in exponential growth at cell densities greater than 10-fold higher than the control cultures. Anchorage-independent growth of normal human cells is also promoted by PDD in a dose-dependent manner, with prior subculturing in the presence of PDD being required for maximal colony formation. The structural analog 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ -PDD) fails to elicit similar cellular responses.

KEY WORDS: Phorbol esters, tumor promoters, cultured human cells, increased saturation density, anchorage-independent growth, mimicry of transformation.

Investigations of staging of carcinogenesis, based on the two-stage mouse skin model (Berenblum, 1975), have been reported with various cell culture systems (Blumberg et al., 1976; Colburn et al., 1979; Fisher et al., 1979; Ishii et al., 1978; Mondal et al., 1976; Rovera et al., 1977). Tumor promoters elicit numerous changes in cell growth characteristics in these systems even in the absence of initiation of carcinogenesis; an in vitro phenomenon sometimes referred to as mimicry of transformation (Weinstein et al., 1979). However, there have been few reports of phorbol ester tumor promoters significantly altering the growth properties of normal human cells in vitro, thereby greatly impeding the study of promotion of carcinogenesis as it relates specifically to man. A slight decrease in the size of diploid human fibroblasts induced by phorbol esters was reported to allow a marginal increase (up to 50%) in the saturation density attainable under standard cell culture conditions (Diamond et al., 1974). At low cell densities, phorbol esters reportedly inhibit the proliferation of normal human cells which is in contrast to the expected in vitro response for a tumor promoter (Gansler and Kopelovich, 1981). The tumor promoters do induce various changes in the in vitro growth characteristics of mutant human fibroblasts and human cancer cells that are more characteristic of promotion as defined for cultured rodent cells (Gansler and Kopelovich, 1981; Kinzel et al., 1981; Kopelovich et al., 1979; Liebermann et al., 1981; Rovera et al., 1979).

The expression of transformed phenotypes was reported by Milo et al. (1981) to be augmented by growth of human skin fibroblasts in culture medium supplemented with excess (8X) nonessential amino acids subsequent to treatment with chemical or physical carcinogens. While examining the long-term effects of the tumor promoter PDD on normal human cells in culture, it was observed that significant changes in phenotypic expression were obtained in the same medium. Partial characterization of PDD-induced changes in the growth properties of diploid human cells in vitro is the subject of this report.

## MATERIALS AND METHODS

### Cell culture

Primary cultures of normal human cells were prepared from neonatal foreskin as described by Riegner et al. (1976). At the first passage of the primary culture, the fetal bovine serum (Reheis, Kankakee, IL, or Sterile Systems, Logan, UT) content in Eagle's minimal essential medium (MEM) (GIBCO, Grand Island, NY) was reduced from 20% to 10%, and it was maintained at that level for subsequent passages. The MEM was also supplemented with 1 mM sodium pyruvate, gentamicin (50 µg/ml) 0.2% sodium bicarbonate, 2X vitamins, and either 8X nonessential amino acids or 1X nonessential amino acids plus 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. Addition of phorbol esters to matched cultures was initiated at the first passage of the primary culture. Post-confluent cell populations in duplicate were trypsinized, and cells were counted with a hemacytometer to establish saturation densities. For the determination of anchorage-independent growth, cell culture dishes (60 mm) were overlaid with 5 ml of 1.0% Difco agar in MEM supplemented with 20% fetal bovine serum, 20% tryptose phosphate broth, 1 mM sodium pyruvate, 1X nonessential amino acids, 2X vitamins, and 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. The cells ( $10^5$ ) in single cell suspensions were overlaid in duplicate in 10 ml of 1.2% methylcellulose in S-MEM (MEM for suspension culture) supplemented as above, but without tryptose phosphate broth. Colonies were scored after 21 days.

### Phorbol esters

PDD and 4 $\alpha$ -PDD (P-L Biochemicals, Milwaukee, WI) were dissolved in acetone. The acetone concentration was 0.01% in phorbol treated and untreated cultures.

## RESULTS AND DISCUSSION

### Increased saturation density

That  $10^{-7}$ M PDD was able to promote a significant increase in the saturation density of normal human cells cultured in the medium containing 8X nonessential amino acids is shown in Figure 1A. The saturation density for the normal control cells was approximately  $4 \times 10^4$  cells  $\text{cm}^{-2}$  at most passages, while the PDD treated cells maintained levels 2 to 4-fold higher for most of the lifetime of the cultures. The specificity of the effect for an active tumor promoter is demonstrated by the fact that  $4\alpha$ -PDD gave results similar to the untreated controls. A sustained PDD-induced elevation in saturation density of comparable magnitude has been obtained with four different primary cultures in medium supplemented with 8X nonessential amino acids. This 2 to 4-fold increase in saturation density was obtained in spite of the fact that  $10^{-7}$ M PDD reduced the cloning efficiency 20 to 30% with all four primary cultures under the conditions employed.

While examining the effects on saturation density of supplementation with various other combinations and concentrations of amino acids, an even greater initial increase was observed for normal cells treated with  $10^{-7}$ M PDD in medium containing 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine (Figure 1B). Cell densities in the PDD treated cultures reached levels 7-times those of the acetone and  $4\alpha$ -PDD controls. Similar results have been obtained with eight different primary cultures, with the increases in saturation density ranging from 5 to 10-fold. Substituting bovine serum for fetal bovine serum in the culture medium for the duration of the experiment did not alter the results significantly (data not presented). Although the timing of the transient increase in density has been somewhat variable, all the cultures have exhibited a sustained 2 to 4-fold elevation subsequently, similar to that seen

in 8X nonessential amino acids. Removal of PDD from the culture medium resulted in a return to near-normal saturation density within a few population doublings (Figure 1B). Although it was suggested that smaller cell size ( $\leq 30\%$ ) might explain the phorbol ester-induced increases in saturation density ( $\leq 50\%$ ) reported by Diamond et al. (1974), it obviously could not explain the increases of up to 1,000% in the present investigation. Our PDD treated cells did appear somewhat smaller by microscopic observation, but no significant PDD induced changes in cell size were determined using either Coulter counter sizing or packed cell volume. The cells in treated and untreated cultures also retained predominantly a fibroblast-like morphology.

The degree to which PDD caused a loss of sensitivity to contact inhibition of cell division of normal human cells is demonstrated in Figure 2 as well. Cells treated with  $10^{-8}$ M PDD remained in exponential growth at significantly higher ( $>10$ -fold) cell densities than untreated cells. Again, this is in contrast to the reported phorbol ester-induced inhibition of cell proliferation for sparse cultures of normal human cells under standard culture conditions (Gansler and Kopelovich, 1981). However, as with most studies involving cultured human cells, the phorbol ester utilized previously was 12-O-tetradecanoylphorbol-13-acetate (TPA), and the treatment period was short-term. TPA has been reported both to inhibit and to stimulate proliferation of mouse fibroblasts depending on the culture conditions and timing employed (Tomei et al., 1981). Therefore, chronic exposure to the more stable phorbol ester PDD in culture medium supplemented with increased concentrations of amino acids might explain the incongruities for saturation density compared to other human cell studies.



### Anchorage-independent growth

PDD was also able to promote anchorage-independent growth of the normal human cells, and a typical cell colony in methylcellulose is shown in Figure 3. Anchorage-independent growth is not a common tumor promoter-induced phenomenon for normal rodent cells or normal human cells under standard culture conditions (Antecol and Mukherjee, 1982; Colburn et al., 1979; Fisher et al., 1979; Kopelovich et al., 1979), i.e., it is not a common characteristic of mimicry of transformation for normal cells (Weinstein et al., 1979). However, some carcinogen-initiated mouse epidermal cell lines and adenovirus-transformed rat embryo fibroblasts do exhibit increased anchorage-independent growth in the presence of phorbol ester and non-phorbol ester tumor promoters, and the response occurs in a dose-dependent manner (Colburn et al., 1979; Fisher et al., 1979 and 1982).

If the normal human skin cells were exposed to PDD continuously under the prescribed conditions, anchorage-independent growth was also promoted in a dose-dependent fashion (Figure 4). As shown, few colonies were obtained when the cells were passaged only 2 times (ca. 7 population doublings) with PDD prior to seeding in methylcellulose, while the number increased significantly after 4 passages (ca. 14 population doublings). The need for prior subculturing in the presence of PDD to obtain optimal anchorage-independent growth can be seen in Figure 5 as well. When parallel cultures of control cells and cells exposed to  $10^{-8}$ M PDD for approximately 20 population doublings were seeded in methylcellulose containing  $10^{-7}$  PDD, the cells with prior exposure to the tumor promoter exhibited significantly enhanced colony formation. In all cases, the few human cell colonies obtained without added promoter were much smaller than those with promoter. The latter observation is similar to that reported previously for transformed rodent cells (Fisher et al., 1979).

Further changes in the cell culture environment may make it possible to obtain even greater PDD-induced alterations in the growth properties of human diploid cells. However, variations in amino acid concentrations in the culture medium appear to be important to obtain significant effects. In addition, starting the phorbol ester treatment at the first passage of the primary culture may enhance the observable cellular responses. The in vitro transformation protocol of Milo et al. (1981) utilizing the same cell type, requires that the cells undergo few population doublings before carcinogen treatment in order to attain neoplastic transformation. Therefore, human cell populations may become refractory to initiation and promotion of carcinogenesis with prolonged culture, i.e., they are modulated by the tissue culture environment. The different treatment protocol utilized might thus explain the significantly greater phenotypic response to a phorbol ester tumor promoter than demonstrated previously for normal human cells. Using this new protocol, it may be possible to study promotion of carcinogenesis with normal human cells in vitro.

#### ACKNOWLEDGMENTS

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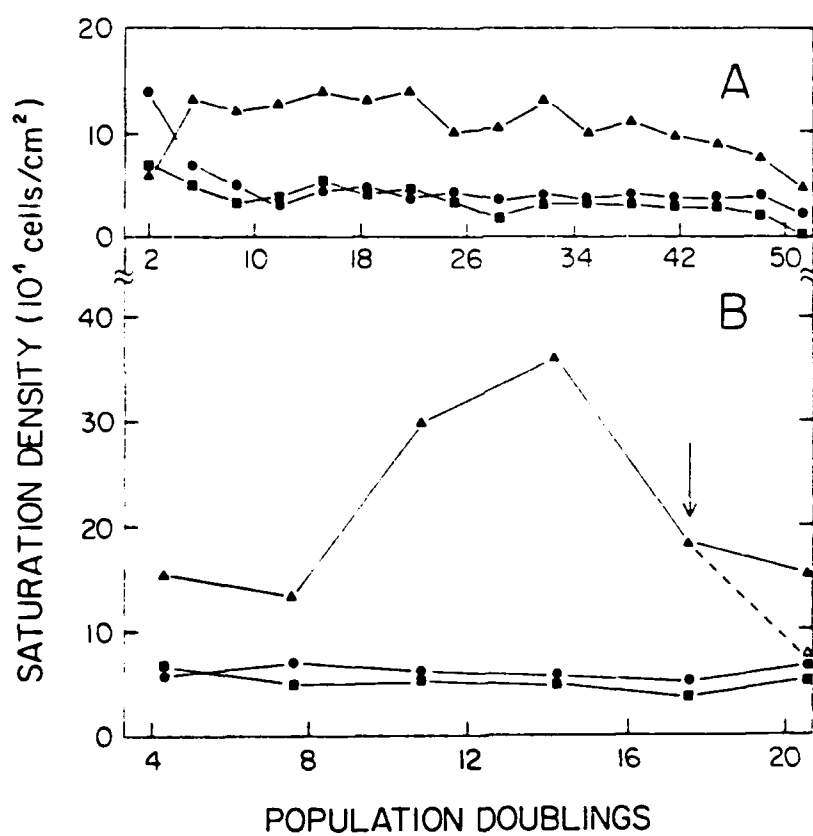
Figure 1. Saturation densities of human diploid cells exposed continuously to phorbol esters. The cell populations are: Control (●),  $10^{-7}$ M PDD (▲), and  $10^{-7}$ M  $4\alpha$ -PDD (■). (A) Human diploid cells cultured in medium supplemented with 8X nonessential amino acids. The primary culture was split at a ratio of 1:4 after which the treated and untreated populations were subcultured at a ratio of 1:10 at 7 day intervals. (B) Human diploid cells cultured in medium supplemented with 1X nonessential amino acids plus 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. Cell populations were subcultured at a ratio of 1:10, were fed with fresh medium after 3 days, and were enumerated after 7-8 days. The arrow indicates the subculture at which PDD was removed from duplicate cell populations (Δ;dashed line).

Figure 2. Growth curves for human diploid cells grown in the absence and presence of PDD. The cell populations are: Control (●) and  $10^{-8}$ M PDD (▲). Secondary post-confluent cultures (cultures having been exposed to 0.01% acetone  $\pm$   $10^{-8}$ M PDD for one passage) in medium supplemented with 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine were subcultured 1:10 (Control) and 1:30 (PDD) on day 0. Duplicate cultures were trypsinized and counted with a hemacytometer each day.

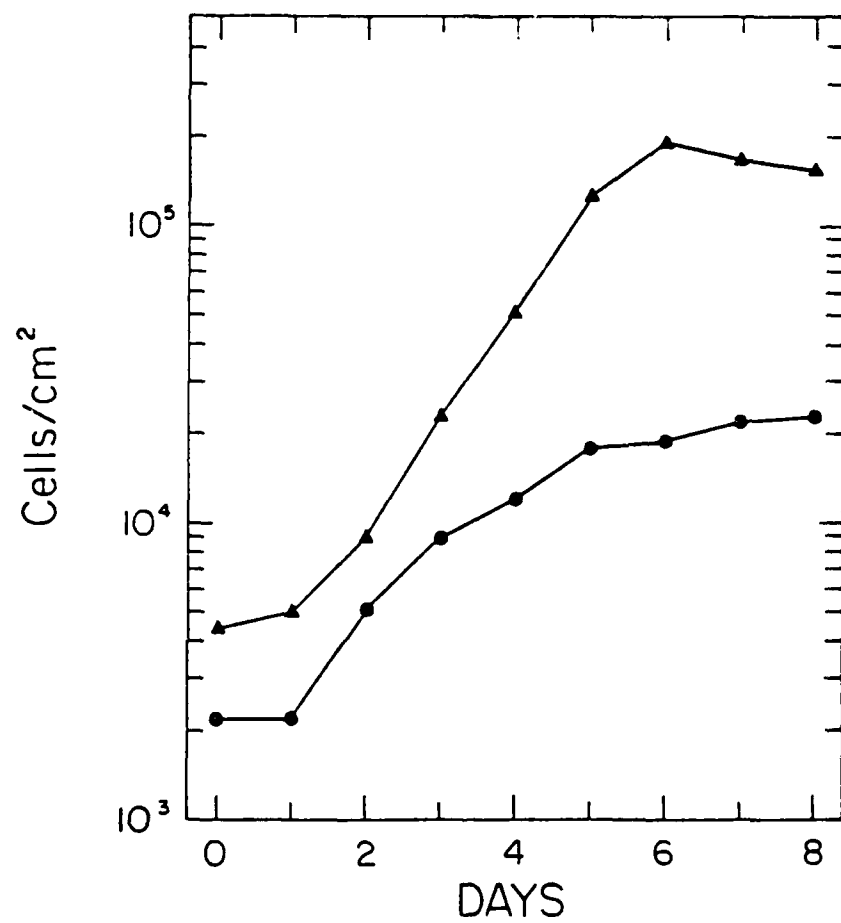
Figure 3. Photomicrograph of a PDD-induced colony of human cells in methylcellulose. Conditions as described in MATERIALS AND METHODS, with the overlay medium supplemented with  $10^{-7}$ M PDD.

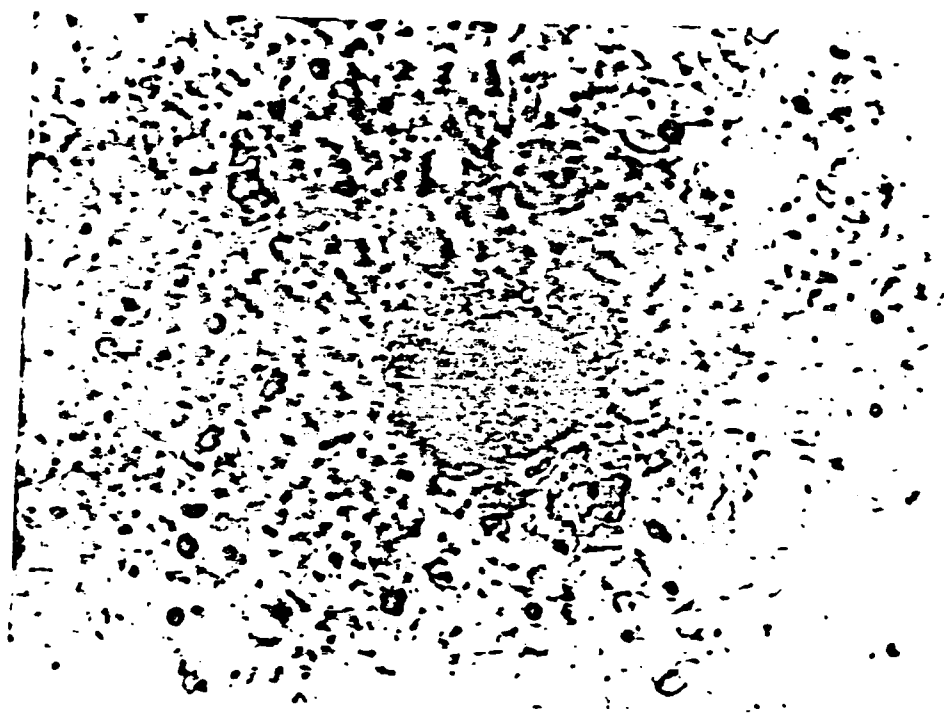
Figure 4. Dose dependence of PDD-induced anchorage-independent growth of normal human cells. The cells were seeded in methylcellulose after two passages (O) and four passages (●) in the indicated concentrations of PDD. Conditions as described in Figure 1B for growth and subculturing of cells and as in MATERIALS AND METHODS for assessing anchorage-independent growth. The methylcellulose overlay medium was supplemented with the indicated concentrations of PDD.

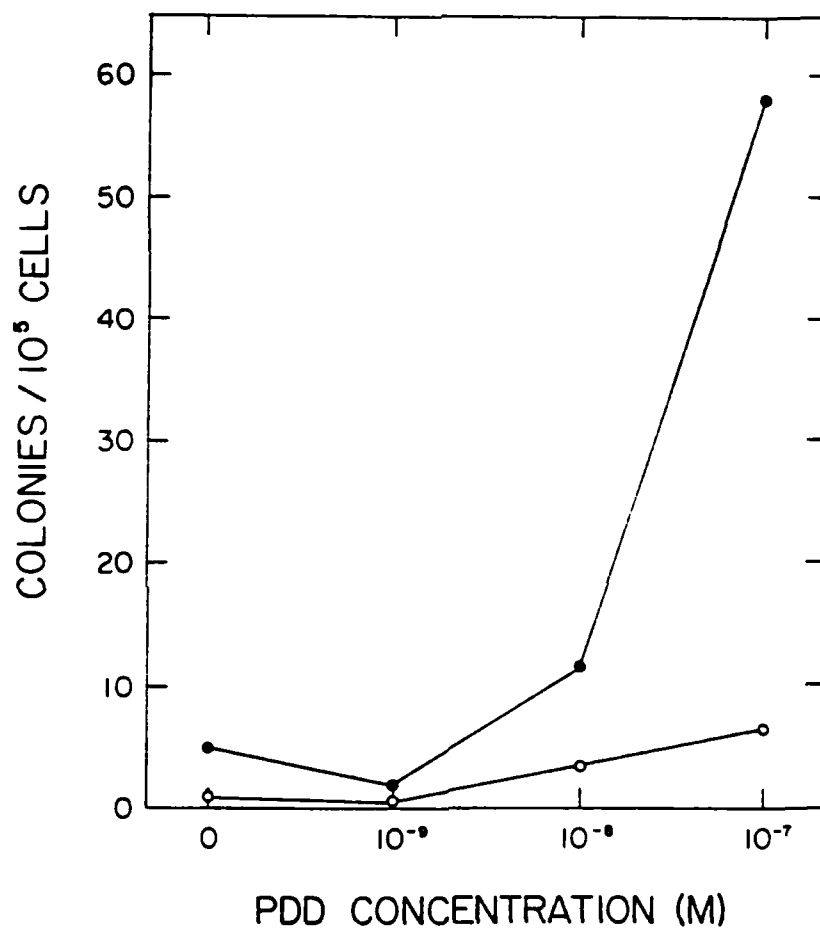
Figure 5. Effect of subpassaging in PDD on anchorage-independent growth of normal human cells. A. The stippled bar indicates the results for cells treated with  $10^{-8}$ M PDD for six passages prior to plating in methylcellulose containing  $10^{-8}$ M PDD. B. The solid bar indicates the results obtained if the same cells (A) were plated in methylcellulose containing  $10^{-7}$ M PDD. C. The stippled bar indicates the results for parallel untreated control cells (derived from the same primary culture) plated in methylcellulose without PDD. D. The solid bar indicates the results obtained if the same cells (C) were plated in methylcellulose containing  $10^{-7}$ M PDD. Culture conditions as described in Figure 1B and MATERIALS AND METHODS.

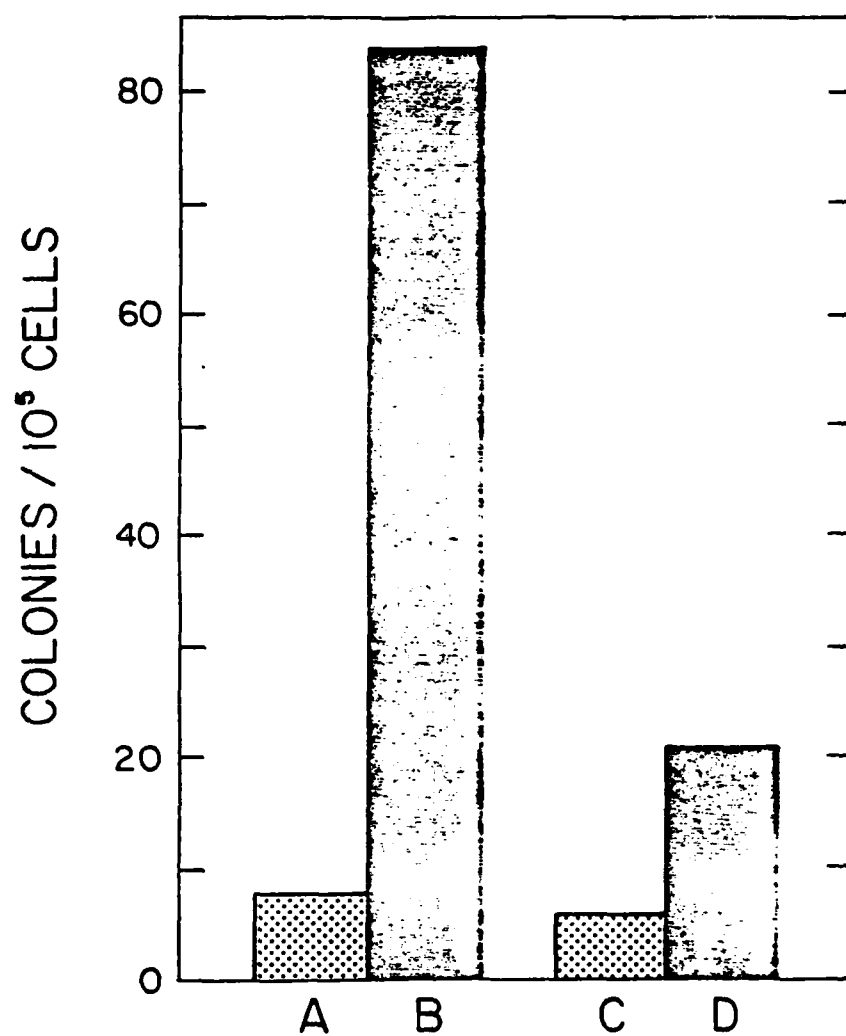












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PUTATIVE TUMOR PROMOTER RESPONSE IN CULTURED HUMAN DIPLOID CELLS. Ronald W. Trewyn, Holly B. Gatz, Lani A. Davakis, and Mark S. Elliott. Ohio State University, Columbus, Ohio 43210.

Investigations of the role of altered RNA metabolism in carcinogenesis have dictated the need for a human cell culture model to study the stages subsequent to initiation. By proper supplementation of the culture medium to optimize for changes in tRNA modification, carcinogen-initiated human skin cells exhibit a 3 to 4-fold increase in saturation density when exposed continuously to the tumor promoter phorbol 12,13-didecanoate (PDD). The increase in saturation density promoted by PDD does not require carcinogen initiation, but an active phorbol ester tumor promoter is required. Treatment of normal diploid cells for only 6 population doublings with PDD gives a significant increase in saturation density, whereas treatment with the inactive analog 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ -PDD), actually decreases the saturation density somewhat. For promotion of growth in soft agar,  $\beta$ -naphthylamine-initiated human cells appear to require the continuous presence of PDD. In this case, exposure to PDD is started 1 to 2 population doublings after carcinogen treatment. The human cells exposed to PDD exhibit aberrant tRNA methyltransferase activity, and the tRNA isolated from these cells is hypomodified for quinine [7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine], a serum-derived purine. Similar tRNA changes are not promoted by 4 $\alpha$ -PDD.

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